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Detection Method

The invention relates to a method for quantitative or qualitative detection of an analyte in a sample.

For detection of analytes, various assay methods are used, e.g., the so-called sandwich immunoassays. In such an immunoassay, two antibodies bind to two epitopes of the analyte to be detected, forming a sandwich complex.

In a heterogeneous sandwich immunoassay, one antibody is bound to a solid phase (e.g., a microtitration plate, magnetic particle, etc.) and serves to separate the sandwich complex from the liquid phase, while the other antibody carries a detectable label (e.g., an enzyme, a fluorescence label or a chemiluminescence label, etc.) for detection of the immune complex. These assay methods are further divided into so-called one-step sandwich immunoassays, in which the two antibody reagents are incubated simultaneously with the sample, and two-step sandwich immunoassays, in which the sample is incubated first with the solid-phase reagent and then, after a separation and washing step, the antibody-antigen complex bound to the solid phase is incubated with the detection reagent.

In a homogeneous sandwich immunoassay, e.g., a nephelometric latex assay, the antibody reagents are incubated and measured together with the sample, but no separation or washing step is performed at any point in the method. In other words, there is no separation of the antibody-bound analyte from the free analyte.

The one-step immunoassay, like the homogeneous sandwich immunoassay, has the advantage that it can be performed more rapidly and is more easily automated. However, a fundamental problem occurs with these assay methods when the analyte concentration in the sample is very high. Very high sample concentrations are known, e.g., of analytes such as albumin, immunoglobulins, β2-microglobulin, human chorionic gonadotropin (hCG), ferritin, α-fetoprotein (AFP). If unbound analyte is not removed before incubation with the labeled antibody, as is done in the two-step sandwich immunoassay, it may happen that the

binding sites of the antibodies become saturated without forming a sandwich complex. This results in the measuring signal initially increasing in the case of samples with an increasing analyte concentration and then decreasing again after a certain limit concentration (see, for example, Figure 1 in Papik et al. (1999), Clin. Chem. Lab. Med. **37**:471-476). This phenomenon, also known as the "prozone effect" or "high-dose hook effect" and referred to hereinafter as the "hook effect" results in the fact that the measuring signal of samples having a very high analyte concentration is found within the standard curve range, and a sample concentration that is much too low is assigned to these samples incorrectly.

It is therefore important to design the one-step immunoassay and/or the homogeneous sandwich immunoassay in such a way that the limit concentration is shifted as far as possible into the high analyte concentration range or, better yet, beyond the naturally occurring analyte concentration range. Another and/or an alternative measure is to introduce a method step that indicates a hook sample, so that the analyte concentration can be determined after appropriate sample dilution.

To solve this problem, Papik et al. propose analysis of the reaction kinetics by a very complex computation method in a turbidimetric assay.

EP-A1-0 787 986 proposes that polyclonal antibodies purified by affinity chromatography should be used in such assays. However, this method has the disadvantage that it cannot be used with all assay methods, in particular not with non-immunochemical assay methods. Furthermore, polyclonal antibodies are not available for all analytes to be detected.

US 4,590,169 and US 4,595,661 describe the use of antibodies, each of which has a different affinity, to reduce the hook effect. According to US 4,595,661, the sample is incubated with an antibody bound to a polystyrene bead and two horseradish peroxidase-antibody conjugates, wherein the three antibodies bind to the different epitopes on the protein to be detected and the two enzyme-labeled antibodies have a distinctly different affinity from one another. After incubation, the unbound substances are removed by washing and the enzyme activity bound to the polystyrene bead is measured. One disadvantage of these methods is that antibodies of different affinities are needed. Another disadvantage is the reduced detection sensitivity of such assays because the use of

conjugates with antibodies having a low affinity results in an increase in the background signal because of the higher non-specific binding to the solid phase.

EP-A2-0 617 285 describes a homogeneous turbidimetric hCG assay, in which to reduce the hook effect, the sample is first incubated with a soluble anti-hCG antibody fragment having at least two binding sites for the analyte, then latex-particle-bound antibody fragments of anti-hCG antibodies having a different epitope specificity are added and the change in absorbance is measured. Addition of the unlabeled soluble antibody produces an additional crosslinking of the latex particles and therefore causes a shift in the hook effect in the direction of higher analyte concentrations. However, homogeneous turbidimetric measurement methods have the disadvantage that they do not have the detection sensitivity required for certain parameters.

For those skilled in the art, the object is therefore to develop detection methods, in particular methods having a high detection sensitivity, so that a hook effect is prevented, reduced and/or at least detected.

This object is achieved by providing the inventive method according to Claim 1. In this method for quantitative or qualitative detection of an analyte A in a sample for detection, prevention and/or reduction of the hook effect, the sample is incubated with an analyte A-specific binding partner R1, which is associated with a solid phase, an analyte A-specific binding partner R2, which is associated with a label L1, and an analyte A-specific binding partner R3, which is associated with a label L2. The binding partners R2 and R3 are selected so that saturation of the analyte A binding sites of the binding partners R2 in the incubation batch occurs at a higher analyte A concentration and/or at a later point in time during incubation in comparison with saturation of the analyte A binding sites of the binding partners R3 present in the incubation batch. The measuring signal, which depends on L1, is either separated in time from the measuring signal that depends on L2 or on L1 plus L2, or is determined with the help of another measurement method. The measuring signal in each case is preferably measured by labels associated with the sandwich complexes thus formed.

The inventive assay method may be performed independently of the order in which R1, R2 and R3 are added or of the respective incubation time of the sample and the respective

binding partner. Unbound reagent components and/or sample constituents may also be removed by a separation or washing step before the measuring signal(s) is/are measured. Such a separation and/or washing step may also be performed, for example, after measurement of the measuring signal which depends on L1 or before measurement of all measuring signals.

The measuring signals may be detected, e.g., as the individual measured value, the mean, median or sum of multiple individual measured values and/or in the form of kinetics. Different measurement methods are understood to refer to methods capable of detecting the L1-dependent measuring signal independently of the L2-dependent or L1-plus-L2-dependent measuring signal. For example, the label L1 may be a microparticle, which can be measured by a nephelometric method, and the label L2 may be an enzyme whose activity is determined photometrically, for example. In another assay method, L1 and L2 may be two fluorescence labels, the fluorescence of each being measured at a different wavelength. For further analysis, for example, the ratio between the "L1 value" and the "L2 value" and/or the "L2-plus-L1 value" may be formed, with the term "value" may being understood to refer directly to the measuring signal or a value proportional to the measuring signal and further revised on the basis of the measuring signal. The ratio thereby obtained may be compared with characteristic quantities that are specific for the assay, so that the person or automatically controlled machine performing the assay is able to detect rapidly whether there is a hook effect and/or whether the sample should be measured again in a suitable dilution.

This method is based on the fact that the formation of the sandwich complexes R1-A-R2 and R1-A-R3 is influenced by the hook effect to different extents. A declining measuring signal occurs only at a higher analyte concentration in the sample with the sandwich complex formation R1-A-R2 in comparison with the sandwich complex formation R1-A-R3. Suitable binding partners and reaction conditions can be ascertained relatively easily by those skilled in the art by first measuring the sandwich complex formation R1-A-R2 and the sandwich complex formation R1-A-R3 in separate assay methods.

In a preferred embodiment of the invention, the binding partner R2 and R3 is the same binding partner. R2 is used here in the form of R2 aggregates and/or many R2 molecules,

which are associated with a suspendable solid phase, whereas R3 is used as a solitary molecule at least in the initial phase of incubation.

R2 and R3 may also be different binding partners. For example, one binding partner may be used as R2, which is directed against an individually occurring binding site on the analyte, whereas R3 recognizes a multiply occurring binding site on the analyte. Furthermore, a binding partner R2 whose ability to bind to the analyte is weaker than that of the binding partner R3 may be used.

As a rule, the detection sensitivity, i.e., the lowest analyte concentration that can still be detected reliably, of the assay system of solid phase-R1 and label-R2 is lower than the detection sensitivity of the assay system of solid phase-R1 and label-R3. However, the solid phase-R1 and label-R2 assay system will still indicate correct concentration values even at a higher analyte concentration. In other words, the "solid phase-R1 and label-R2" assay system covers the upper measurement range better, and the "solid phase-R1 and label-R3" assay system covers the lower measurement range better. Through separate detection of the L1 measuring signal, which is proportional to the R1-A-R2 complex formation, from the L2-dependent or L1-plus-L2-dependent measuring signal, it is possible to expand the measurement range of the inventive assay method and/or detect a hook effect. For example, if the L1 measuring signal is above the highest standard curve value and the L2 measuring signal is within the standard curve, also known as a calibration curve, this is a reliable indication of a "high-dose hook sample." In the case when a hook effect is detected, the sample is assayed again in an appropriate dilution and the correct analyte concentration is determined.

In contrast with the methods known previously, the inventive method also has a broad applicability beyond the field of immunoassays, e.g., in binding assays with nucleic acid molecules as specific binding partners. With the inventive method, neither specially purified antibodies nor antibodies having a different affinity are required. On the contrary, in a preferred variant of the inventive method, R2 and R3 are the same specific binding partner. Furthermore, the inventive method can be automated more easily.

Another advantage of the inventive method is the possibility of expanding the measurement range. For example, the L1-dependent measuring signal may be utilized to

ascertain the concentration of samples with a higher concentration, whereas the L2-dependent measuring signal may be utilized to ascertain the concentration of samples with a low concentration. Consequently, fewer samples need be diluted.

Through the corresponding choice of L1 and L2 and/or the other parameters of the method, e.g., the R2 and/or R3 concentration in the incubation batch, it is possible to optimize the inventive method in such a way that, despite the large measurement range and reliable detection of the hook effect, the non-specific solid-phase binding of R2-L1 does not influence the assay sensitivity, which is ensured via the R1-solid phase and R3-L2 [assay] system.

The inventive method may also be used to particular advantage with analytes which are very difficult to determine with a homogeneous and/or one-step sandwich assay because of analytes cross-reacting with the specific binding partners. For example, it is customary to determine LH (luteinizing hormone) with an immunoassay using antibodies to the α-chain and antibodies to the LH-specific part of the β-chain. If the sample happens to contain a large amount of hCG, in the case of a one-step sandwich assay and/or a homogeneous assay, this may result in all or almost all of the binding sites of the antibodies directed against the α-chain being blocked by hCG molecules. Consequently, the antibody-LH-antibody sandwich complex cannot be formed and the LH sample concentration is determined incorrectly. With the help of the inventive method, it is now possible to measure such analytes not with a two-step sandwich assay, as is customary, but instead with the much faster one-step sandwich assay, in which R1 is selected so that this binding partner detects the analyte and the cross-reacting substances equally well, and R2 and R3 each recognize specifically either the analyte or the cross-reacting substances. To return again to the example described above to illustrate the invention, antibodies directed against the α-chain, for example, would be used as R1 according to this invention, while specific anti-hCG antibodies would be used as R2 and specific anti-LH antibodies would be used as R3. The L1 signal, which depends on the R1-hCG-R2 sandwich formation, indicates whether or not the sample must be measured again for correct determination of the LH concentration, e.g., in a two-step immunoassay. This inventive method can also be used accordingly to measure antibodies, in particular for measuring antibodies of a certain immunoglobulin class, e.g., IgM, which are directed against a certain antigen, e.g., viral or bacterial, with the other antibodies present in the sample may being present in a great

excess. This use of the inventive method as described above is to be understood as included in the phrase "for detection, prevention and/or reduction of the hook effect" in the sense of the present invention.

Preferred embodiments of the invention are described in Claims 2 through 19. Before these and other preferred embodiments of the invention are explained in greater detail, a few terms should be explained first to facilitate an understanding of the present invention.

In a "quantitative detection," the amount or concentration of the analyte in the sample is measured. The term "quantitative detection" also includes semiquantitative methods which detect only the approximate quantity or concentration of the analyte in the sample or may be used only for a relative statement of quantity or concentration. A "qualitative detection" is understood to refer to detection of the presence or absence of an analyte in a sample or indicating that the concentration of the analyte in the sample is above or below one or more specific threshold values.

The term "analyte" is understood to refer to the substance to be detected in the assay method. Examples of an analyte are listed on pages 8 to 15 in EP-A2-0 515 194. The analyte may be a member of the specific binding pair. The analyte may have one binding site (monovalent, usually a hapten) or multiple binding sites (polyvalent). In immunochemical assays, such a binding site is frequently also referred to as an epitope. Furthermore, the analyte may be a single substance or a group of substances having at least one single common binding site.

A monovalent analyte usually has a molecular weight of approx. 100 to 2000, in particular 125 to 1000. Many oligopeptides, oligonucleotides, oligosaccharides, pharmaceutical medicines, drugs, metabolites, pesticides, etc. are covered by the term monovalent analyte. A polyvalent analyte usually has a molecular weight of more than 5000, mostly more than 10,000. Examples of polyvalent analytes include polypeptides, polysaccharides, nucleic acids, cells, cell constituents including chromosomes, genes, mitochondria and other cell organelles, cell membranes, etc. The substances to be detected are often proteins. Such proteins may be members of a protein family whose members are characterized by similar structural features and/or by a similar biological function. Examples of protein families that are of interest analytically include proteins of disease pathogens, immunoglobulins,

cytokines, enzymes, hormones, tumor markers, metabolic markers, tissue-specific antigens, histones, albumins, globulins, scleroproteins, phosphoproteins, mucins, chromoproteins, lipoproteins, nucleoproteins, glycoproteins, proteoglycans, receptors, HLA, coagulation factors, myocardial infarction markers, etc. Other substances that are of interest analytically include, for example, single- or double-stranded oligonucleotides and polynucleotides.

A "sample" in the sense of this invention is understood to refer to the material presumably containing the substance ("analyte") to be detected. The term "sample" includes, for example, biological fluids or tissue, in particular from humans and animals, such as blood, plasma, serum, sputum, exudate, bronchoalveolar lavage, lymphatic fluid, synovial fluid, seminal fluid, vaginal mucus, feces, urine, cerebrospinal fluid, hair, skin, tissue samples or sections. Furthermore, it also includes cell culture samples, plant fluids or tissue, forensic samples, water and wastewater samples, foods, pharmaceutical drugs. The samples may be pretreated, if necessary, to make the analyte accessible for the detection method or to remove interfering constituents of the sample. Such pretreatment of samples may include the separation and/or lysis of cells, precipitation, hydrolysis or denaturing of sample constituents, e.g., proteins, centrifugation of samples, treatment of sample with organic solvents, e.g., alcohols, especially methanol, treatment of the sample with detergents. A sample is often transferred to another medium, usually aqueous, which should preferably not interfere with the detection method at all. The analyte may also be amplified. Amplification of nucleic acid leads to generation of one or more copies of the nucleic acid chain to be detected, for example. Those skilled in the art are readily familiar with such amplification methods, e.g., "polymerase chain reaction" (PCR), "ligase chain reaction" (LCR), "amplification using Q beta replicase," "nucleic acid sequence-based amplification" (NASBA), "single primer amplification" (ASPP) and others.

A "specific binding partner" is understood to be a member of a specific binding pair. Members of a specific binding pair are two molecules, each having at least one structure complementary to a structure of the other molecule, the two molecules being capable of binding by binding of the complementary structures. The term "molecule" also includes molecular complexes, e.g., enzymes consisting of apoenzyme and coenzyme, proteins consisting of several subunits, lipoproteins consisting of protein and lipids, etc. Specific binding partners may include naturally occurring substances or those produced, e.g., by

chemical synthesis, microbiological techniques and/or genetic engineering methods. In the meantime, specific binding partners can be selected with the help of phage display libraries, synthetic peptide databases or by recombinatorial antibody libraries (Larrick and Fry (1991), Human Antibodies and Hybridomas, **2**:172-189). The following may be mentioned, for example, to illustrate the concept of specific binding partners, but not to be understood restrictively: thyroxine-binding globulin, steroid-binding proteins, antibodies, antigens, haptens, enzymes, lectins, nucleic acids, repressors, oligonucleotides and polynucleotides, protein A, protein G, avidine, streptavidine, biotin, complement components C1q, nucleic acid-binding proteins, etc. Specific binding pairs include, for example, antibody-antigen, antibody-hapten, operator-repressor, nuclease-nucleotide, biotin-avidine, lectin-polysaccharide, steroid-steroid-binding protein, active ingredient-active ingredient receptor, hormone-hormone receptor, enzyme-substrate, IgG-protein A, complementary oligonucleotides or polynucleotides, etc.

The term "antibody" is understood in the sense of the present invention to include an immunoglobulin, e.g., an immunoglobulin of the class and/or subclass IgA, IgD, IgE, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgG_4, IgM. An antibody has at least one binding site (often called a paratope) for an epitope (often also called an antigenic determinant) on an antigen or hapten. Such an epitope is characterized, e.g., by its three-dimensional structure and/or by the presence of polar and/or apolar groups. The binding site of the antibody is complementary to the epitope. The antigen-antibody reaction and/or the hapten-antibody reaction functions according to the so-called "lock-and-key principle" and is usually specific to a high degree, i.e., the antibodies are capable of differentiating minor deviations in the primary structure, the charge, the spatial configuration and steric configuration of the antigen or hapten. The so-called "complementarity determining regions" of the antibody in particular contribute toward binding of the antibody to the antigen or hapten.

The term "antigens" includes monovalent and polyvalent antigens. A polyvalent antigen is a molecule or a molecular complex to which more than one immunoglobulin can bind at the same time, whereas in the case of a monovalent antigen, only a single antibody can bind to it at a given time. A hapten is usually a molecule is that not immunogenic per se but instead is usually bound to a carrier for immunization purposes.

The term "antibody" is understood in the sense of this invention to refer not only to complete antibodies but also explicitly includes antibody fragments, e.g., Fab, Fv, $F(ab')_2$, Fab' as well as chimeric, humanized, bispecific or oligospecific or single-chain antibodies, as well as aggregates, polymers and conjugates of immunoglobulins and/or fragments thereof if the binding properties to the antigen or hapten are preserved. Antibody fragments can be produced, for example, by enzymatic cleavage of antibodies with enzymes such as pepsin or papain. Antibody aggregates, polymers and conjugates can be generated by a variety of methods, e.g., by heat treatment, reaction with substances such as glutaraldehyde, reaction with immunoglobulin-binding molecules, biotinylation of antibodies and subsequent reaction with streptavidine or avidine, etc.

An antibody in the sense of this invention may be a monoclonal or polyclonal antibody. The antibody may be produced by the usual methods, e.g., by immunization of a person or animal, e.g., mouse, rat, guinea pig, rabbit, camel, horse, sheep, goat, chicken (see also Messerschmid (1996), BIOforum **11**:500-502) and subsequent production of antiserum or by establishing hybridoma cells and subsequent purification of the secreted antibodies or by cloning and expression of the nucleotide sequences and/or modified versions thereof which code for the amino acid sequences that are responsible for binding the natural antibody to the antigen and/or hapten. Antibodies can also be produced, if necessary, with the help of the methods of genetic engineering in plant cells, e.g., yeast cells (Fischer et al. (1999), Biol. Chem. **380**:825-839; Hiatt et al. (1992), Genetic Engineering **14**:49-64), animals cells and prokaryotic cells (see, e.g., WO 95/25172) and isolated human cells.

The term "solid phase" in the sense of this invention includes an object which consists of porous and/or non-porous, usually water-insoluble material and may have a wide variety of forms, e.g., vessel, tube, microtitration plate, sphere, microparticle, rod, strip, filter paper or chromatography paper, etc. As a rule, the surface of the solid phase is hydrophilic or can be hydrophilized. The solid phase may consist of a wide variety of materials, e.g., organic and/or inorganic materials, synthetic, naturally occurring and/or modified naturally occurring materials. Examples of solid-phase materials include polymers, e.g., cellulose, nitrocellulose, cellulose acetate, polyvinyl chloride, polyacrylamide, crosslinked dextran molecules, agarose, polystyrene, polyethylene, polypropylene, polymethacrylate or nylon, ceramics, glass, metals, in particular noble metals such as gold and silver, magnetite,

mixtures or combinations thereof, etc. Cells, liposomes or phospholipid vesicles are also covered by the term "solid phase."

The solid phase may have a coating of one or more layers, e.g., proteins, carbohydrates, lipophilic substances, biopolymers, organic polymers or mixtures thereof to suppress or prevent non-specific binding of sample constituents to the solid phase, for example, or to achieve improvements with regard to the suspension stability of particulate solid phases, for example, the stability in storage, the shape stability or resistance to UV light, microbes or other destructive agents.

The term "associated" is to be understood in a broad sense and comprises, for example, covalent and non-covalent binding, direct and indirect binding, adsorption onto a surface and inclusion in a recess or a cavity, etc. In covalent binding, the antibodies or binding partners are bound to the solid phase or to the label by a chemical bond. It is customary to speak of covalent binding between two molecules if at least one atomic nucleus of one molecule shares electrons with at least one atomic nucleus of the second molecule. Examples of non-covalent binding include surface adsorption, inclusion in cavities or binding of two specific binding partners. In addition to direct binding to the solid phase or the label, the antibodies or binding partners may also be bound to the solid phase or the label indirectly via specific interaction with other specific binding partners (see also EP-A2-0 411 945). This will be illustrated in greater detail on the basis of examples. The biotinylated antibody may be bound to the label via label-bound avidine; or a fluorescein-antibody conjugate may be bound to the solid phase via solid phase-bound anti-fluorescein antibody; or the antibody may be bound to the solid phase or the label via immunoglobulin-binding proteins.

A "signal-forming system" may refer to one or more components, with at least one component being a detectable label. A label may be understood to be any molecule that produces a signal itself or is capable of inducing production of a signal, e.g., a fluorescent substance, a radioactive substance, an enzyme or a chemiluminescent substance. The signal may be detected or measured on the basis of the enzyme activity, the chemiluminescence, the light absorbance, the light scattering, the emitted electromagnetic or radioactive radiation or a chemical reaction.

A "label" is itself capable of generating a detectable signal so that no other components are necessary. Many organic molecules absorb ultraviolet and visible light, and due to the energy transferred through light absorbance, these molecules may enter an excited energy state and emit the absorbed energy in the form of light at a different wavelength than that of the incident light. Other labels may also generate a detectable signal directly, e.g., radioactive isotopes, dyes or magnetic and non-magnetic microparticles.

Other labels in turn require additional components for generating a signal, i.e., the signal-producing system in such a case includes all the components required for forming a signal, e.g., substrates, coenzymes, quenchers, accelerators, additional enzymes, substances which react with enzyme products, catalysts, activators, cofactors, inhibitors, ions, etc.

Suitable labels (see also EP-A2-0 515 194; US 5,340,716; US 5,545,834; Bailey et al. (1987), J. Pharmaceutical and Biomedical Analysis **5**:649-658) include, for example, enzymes such as horseradish peroxidase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, alcohol dehydrogenase, glucose oxidase, β-galactosidase, luciferase, urease and acetylcholinesterase; dyes; fluorescent substances including fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, ethidium bromide, 5-dimethylaminonaphthalene-1-sulfonyl chloride and fluorescent chelates of rare earths; chemiluminescent substances including luminol, isoluminol, acridinium compounds, olefin, enol ethers, enamine, aryl vinyl ether, dioxene, arylimidazole, lucigenin, luciferin and aequorin; sensitizers including eosin, 9,10-dibromoanthracene, methylene blue, porphyrin, phthalocyanine, chlorophyll, rose bengal; coenzymes; enzyme substrates; radioactive isotopes, including ^{125}I, ^{131}I, ^{14}C, ^{3}H, ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{59}Fe, ^{57}Co and ^{75}Se; particles, including magnetic particles, or particles – preferably latex particles – which may themselves be labeled with dyes, sensitizers, fluorescent substances, chemiluminescent substances, isotopes or other detectable labels; sol particles, including gold or silver sols; liposomes or cells, which may themselves be labeled with detectable labels, etc.

The signal-forming system may also comprise components which may enter into a detectable interaction with one another in spatial proximity to one another, e.g., in the form of energy donors and energy receivers such as photosensitizers and chemiluminescent substances (EP-A2-0 515 194), photosensitizers and fluorophores (WO 95/06877), radioactive iodine-125 and fluorophores (Udenfriend et al. (1985), Proc. Natl. Acad. Sci.

82:8672-8676), fluorophores and fluorophores (Mathis (1993), Clin. Chem. **39**:1953-1959) or fluorophores and fluorescence quenchers (US 3,996,345).

An interaction between components is understood to include direct transfer of energy between components, e.g., by light or electron emission and by short-lived reactive chemical molecules. Furthermore, this also includes processes in which the activity of one component is inhibited or enhanced by one or more other components, e.g., inhibition or enhancement of the enzyme activity or inhibition, enhancement or alteration (e.g., wavelength shift, polarization) of the electromagnetic radiation emitted by the component influenced. Interaction among components also includes enzyme cascades. In this case, the components are enzymes, at least one of which supplies the substrate for another, resulting in a maximum or minimum reaction rate of the coupled substrate conversion.

An effective interaction between components usually takes place when they are situated in spatial proximity, i.e., within a distance range of a few µm, in particular within a distance range of less than 600 nm, preferably less than 400 nm, most especially preferably of less than 200 nm.

Microparticles are often used as the solid phase and/or as labels. The term "microparticle" is understood in the sense of this invention to refer to particles having an approximate diameter of at least 20 nm and no more than 20 µm, usually between 40 nm and 10 µm, preferably between 0.1 µm and 10 µm, especially preferably between 0.1 µm and 5 µm, most especially preferably between 0.15 µm and 2 µm. The microparticles may be regularly or irregularly shaped. They may be spheres, spheroids or beads with more or less large cavities or pores. The microparticles may consist of organic or inorganic material or a mixture or combination of the two. They may consist of a porous or non-porous, swellable or non-swellable material. In principle, the microparticles may have any density, but particles having a density approximating the density of water, e.g., approx. 0.7 to approx. 1.5 g/ml are preferred. The preferred microparticles can be suspended in aqueous solutions and form suspensions that are stable for the longest possible duration. They may be transparent, partially transparent or opaque. The microparticles may consist of multiple layers, e.g., the so-called core-and-shell particles having a core and one or more shell layers. The term microparticle includes, for example, dye crystals, metal sols, silica particles, glass particles, magnetic particles, polymer particles, oil droplets, lipid particles,

dextran and protein aggregates. Preferred microparticles are particles that consist of a water-insoluble polymer material and can be suspended in aqueous solution, in particular substituted polyethylenes. Latex particles, e.g., of polystyrene, acrylic acid polymers, methacrylic acid polymers, acrylonitrile polymers, acrylonitrile-butadiene-styrene, polyvinyl acetate-acrylate, polyvinylpyridine, vinyl chloride-acrylate. Latex particles having reactive groups at their surface such as carboxyl groups, amino groups or aldehyde groups which allow covalent binding of, for example, specific binding partners to the latex particles are of particular interest. The production of latex particles is described, e.g., in EP 0 080 614, EP 0 227 054 and EP 0 246 446.

The inventive method is preferably a heterogeneous or homogeneous binding assay according to the sandwich format.

In "homogeneous binding assays" there is no separation between free and complex-bound analytes. Examples of homogeneous immunoassays (see also Boguslaski and Li (1982), Applied Biochemistry and Biotechnology **7**:401-414) include many turbidimetric or nephelometric methods, wherein the specific binding partners used for detection may be associated with latex particles; EMIT® assays; CEDIA® assays; fluorescence polarization immunoassays; luminescent oxygen channeling immunoassays (EP-A2-0 515 194; Ullman et al. (1994), Proc. Natl. Acad. Sci. **91**:5426-5430; Ullman et al. (1966), Clinical Chemistry **42**:1518-1526), etc. With the so-called gene probe assays, the specific binding partners are usually nucleic acid chains that are at least partially complementary to sections of the nucleic acid chain to be detected.

"Heterogeneous binding assays" are characterized by one or more separation steps and/or washing steps. The separation may be accomplished, e.g., by immune precipitation, precipitation with substances such as polyethylene glycol or ammonium sulfate, filtration, magnetic separation, binding to a solid phase, e.g., to a tube, a sphere, a microtitration plate well or filter paper or chromatography paper.

In "heterogeneous binding assays according to the sandwich format," the analyte is usually bound by a solid phase-associated specific binding partner and a specific binding partner associated with one component of a signal-forming system. These may be the same or different specific binding partners, e.g., with a sandwich immunoassay, an analyte-specific

monoclonal antibody may be used as a catcher (e.g., as a solid-phase antibody) as well as being used as a labeled antibody when the analyte has more than one epitope for this antibody. The specific binding partners in the case of a sandwich immunoassay may be analyte-specific antibodies or, if the analyte is itself an antibody, the antigen or a "modified antigen" e.g., a labeled antigen, an antigen fragment or an antigen analog. Examples of such sandwich complexes include: solid phase-antibody<>analyte<>antibody-label or solid phase-antigen<>analyte (= antibody)<>antigen-label.

In the sense of this invention, the "indirect immunoassay" is a special embodiment of a heterogeneous immunoassay. In this case, the analyte is an antibody. One of the specific binding partners is its antigen and/or a modified antigen, and the other specific binding partner is usually an antibody that binds the analyte and/or an immunoglobulin-binding protein. Examples of such complexes, which may be formed in an indirect immunoassay include: solid phase-anti-IgM-antibody<>analyte (= anti-HbsAg-IgM)<>HBsAg label or solid phase-HBsAg<>analyte (= anti-HbsAg-IgG)<>protein A label.

The sandwich assays described above, including the direct immunoassays, may also be performed as homogenous assay methods (see also EP-A2-0 515 194).

A preferred embodiment of the inventive method is characterized in that (i) the sample is incubated with an analyte A-specific binding partner R1, which is associated with a solid phase, an analyte A-specific binding partner R2, which is associated with a label L1, and an analyte A-specific binding partner R3, which is associated with a member X of a specific binding pair; (ii) at a later point in time, label L2, which is associated with the binding pair member Y of a specific binding pair corresponding to X, is added to the assay batch; and (iii) the measuring signal is determined at a minimum of two points in time T1 and T2, with the earlier point in time T1 occurring at the latest shortly after addition of label L2, which is associated with binding pair member Y, and the later point in time T2 occurring after addition of label L2, which is associated with binding pair member Y.

The period of time defined by the term "shortly after addition of label L2" is to be understood as meaning that the period of time "addition of label L2 until measurement of L1" is short in comparison with the period of time " addition of label L2 until measurement

of label L2" i.e., preferably less than 30%, especially preferably less than 15% of the period of time "addition of label L2 until measurement of label L2."

In an especially preferred method, the point in time T1 occurs before the addition of label L2.

Another preferred inventive method is characterized in that (i) the sample is incubated with an analyte A-specific binding partner R1, which is associated with a solid phase, an analyte A-specific binding partner R2, which is associated with a label L1 and an analyte A-specific binding partner R3, which is associated with a member X of a specific binding pair; (ii) at a later point in time, label L2, which is associated with the binding pair member Y of a specific binding pair corresponding to X, is added to the assay batch; and (iii) the measuring signal of L1 and the measuring signal of L2 are determined with the help of different measurement methods.

The label L1 might be, for example a microparticle and the label L2 might be a fluorescence or chemiluminescence label, so that the measuring signal L1 could be determined turbidimetrically or nephelometrically, and the measuring signal of L2 could be determined with the help of a fluorometer or a chemiluminescence meter. In another inventive embodiment, L1 and L2 could be different fluorescence labels whose fluorescent light is detected at another wavelength.

Especially preferred binding pairs X<>Y for the methods described above include in particular biotin<>avidine, biotin<>streptavidine or hapten<>anti-hapten antibodies, e.g., fluorescin<>anti-fluorescin, digoxin<>anti-digoxin or antigen<>anti-antigen antibodies such as peroxidase<>anti-peroxidase or nucleic acid pairs.

An especially preferred inventive embodiment is an assay based on the LOCI™ method in which the sample is incubated with an analyte A-specific binding partner R1, which is associated with a sensitizer particle, an analyte A-specific binding partner R2, which is associated with a chemiluminescence particle, and an analyte A-specific binding partner R3, which is associated with a member X of a specific binding pair, preferably biotin; (ii) at a later point in time, chemiluminescer particles associated with the binding pair members [sic; member] Y, preferably streptavidine and/or avidine, of a specific binding pair, corresponding to X, is added to the assay batch; and (iii) the measuring signal is

determined at a minimum of two points in time T1 and T2, with the earlier point in time T1 being at the latest shortly after addition of the chemiluminescer particles which are associated with the binding pair members Y, and the later point in time T2 being after addition of chemiluminescer particles associated with binding pair members Y. In the excited state, the sensitizer molecules associated with the sensitizer particle can generate singlet oxygen. This singlet oxygen may react with the chemiluminescence compounds associated with the chemiluminescer particles, with the metastable compound thereby formed disintegrating again, producing a flash of light. Because singlet oxygen is stable for only a short period of time in aqueous solutions, only chemiluminescer particles in direct proximity to the sensitizer particles excited by light, e.g., by forming an immune complex, for example, are excited to emit light. The wavelength of the emitted light to be measured can be varied through appropriate fluorescent dyes in the chemiluminescer particles. A detailed description of the LOCI™ method can be obtained from EP-A2-0 515 194, for example. The term "sensitizer particle" is understood to refer in particular to microparticles labeled with one or more dyes which generate singlet oxygen when exposed to light. The term "chemiluminescer particle" is understood to refer in particular to microparticles containing dyes which react with singlet oxygen to emit light.

In the inventive methods, R1 and R2, R1 and R3, R1, R2 and R3 or R2 and R3 may be the same binding partners; this is true in particular when the analyte to be detected has at least two identical binding sites. For example, many bacterial antigens or many tumor markers have repetitive epitopes, so that multiple exemplars of a monoclonal antibody are capable of binding to these antigens. Consequently, with the inventive assay method for detection of such antigens, such a monoclonal antibody may be used as binding partners "R1 and R2," "R1 and R3," "R2 and R3" or "R1, R2 and R3." In a particularly preferred embodiment, R2 and R3 are the same binding partners, but R2 should preferably be associated with a suspendable solid phase.

In the inventive methods, L1 and L2 may also be the same labels, e.g., chemiluminescer particles. Thus in such a case, the measuring signal of the chemiluminescer particles as label L1 could be determined turbidimetrically or nephelometrically, and the measuring signal of the chemiluminescer particles as label L2 plus L1 could be determined with the help of a luminometer. In another embodiment, the measuring signal of the chemiluminescer particles as label L1 and the measuring signal of the chemiluminescer

particles as label L2 plus L1 could each be determined at different points in time with the help of a luminometer.

In an especially preferred inventive method, the solid phase is a suspendable solid phase, preferably microparticles, e.g., latex particles or magnetic particles.

The suspendable solid phase, in particular when they are microparticles, may also function as labels. An especially preferred solid phase would be, for example, latex particles, magnetic particles or sensitizer particles.

Especially preferred is an inventive method in which the binding partners R2 are associated with a suspendable solid phase, in particular when they are covalently bound to the same. The suspendable solid phase includes in particular microparticles, especially preferably microparticles that form the label L1 or most especially preferably chemiluminescer particles.

The preferred inventive method is characterized in that due to the formation of a sandwich, components of a signal-forming system including label L1 and/or label L2 are brought to within a distance of one another which allows an interaction, in particular an energy transfer between these components, and the extent of interaction is measured, e.g., the method described above and in the example based on the LOCI™ technology.

In a most especially preferred inventive method, the signal-forming system also comprises sensitizers associated with microparticles and chemiluminescent substances associated with microparticles.

Furthermore, the invention comprises the use of an analyte A-specific binding partner R1 that is associated with a solid phase, an analyte A-specific binding partner R2 that is associated with a label L1 and an analyte A-specific binding partner R3 that is associated with a label L2 for detection, prevention and/or reduction of the hook effect in a method, in particular in a homogeneous sandwich assay, for quantitative or qualitative detection of an analyte A in a sample. Saturation of the analyte binding sites of the binding partners R2 which are present in the incubation batch occurs here at a higher analyte A concentration and/or at a later point in time during incubation in comparison with saturation of the analyte A binding site of the binding partners R3 in the incubation batch, and the L1-

dependent measuring signal is determined by the L2-dependent or L1-plus-L2-dependent measuring signal either at separate points in time or with the help of another measurement method.

The invention also comprises the use of an analyte A-specific binding partner R1, which is associated with a solid phase, an analyte A-specific binding partner R2, which is associated with a label L1, an analyte A-specific binding partner R3, which is associated with a member X of a specific binding pair, and a label L2, which is associated with the binding pair member Y corresponding to X of a specific binding pair for detection, prevention and/or reduction of the hook effect in a method, in particular a homogeneous sandwich assay, for quantitative or qualitative detection of an analyte A in a sample. Saturation of the analyte A binding sites of the binding partners R2 present in the incubation batch occurs at a higher analyte A concentration and/or at a later point in time during incubation in comparison with saturation of the analyte A binding sites of the binding partners R3 present in the incubation batch.

Another component of the invention is an assay kit for a heterogeneous or homogenous sandwich assay for quantitative or qualitative detection of an analyte A in a sample, characterized in that it contains an analyte A-specific binding partner R1, which is associated with a solid phase, an analyte A-specific binding partner R2, which is associated with a label L1, an analyte A-specific binding partner R3, which is associated with a label L2, preferably in separate containers, and in the sandwich assay, saturation of the analyte A binding sites of the binding partners R2 present in the incubation batch occurs at a higher analyte A concentration and/or at a later point in time during incubation in a comparison with saturation of the analyte A binding sites of the binding partners R3 present in the incubation batch.

Another inventive assay kit for a heterogeneous or homogeneous sandwich assay for quantitative or qualitative detection of an analyte A in a sample is characterized in that it contains an analyte A-specific binding partner R1, which is associated with a solid phase, an analyte A-specific binding partner R2, which is associated with a label L1, an analyte A-specific binding partner R3, which is associated with a member X of a specific binding pair and the label L2, which is associated with the binding pair member Y of a specific binding pair corresponding to X, preferably in separate containers, and whereby in the

sandwich assay, the saturation of the analyte A binding sites of the binding partners R2 present in the incubation bath occurs at a higher analyte A concentration and/or at a later point in time during incubation in comparison with saturation of the analyte A binding sites of the binding partners R3 present in the incubation batch. Such an assay kit is especially preferred when the analyte A-specific binding partner R2, which is associated with a label L1, and the analyte A-specific binding partner R3, which is associated with a member X of a specific binding pair, are present together in one container.

Furthermore, the invention also relates to assay kits containing the components needed to perform the inventive method.

The inventive assay kits may also contain a package insert, dilution buffer, standards, controls, system reagents and/or other components needed to perform the assay. Especially preferred inventive assay kits contain a package insert describing the inventive method.

Figure **1** shows a schematic diagram of a preferred inventive assay method ("F" = solid phase). In a first step, solid-phase-R1 analyte ("A" if present in the sample), R2-L1 and R3-L2 (or R3-X) are mixed together, and this incubation batch is incubated until the point in time T1.

At the point in time T1, the measuring signal of the label L1 contained in the solid-phase-R1-analyte-R2-L1 binding complex is determined. Then Y-L2 (only in the case of R3-X) is added and the incubation batch is incubated until the point in time T2. Then the measuring signal of the label L2, which is present in the solid-phase-R1-analyte-R3-X-Y-L2 binding complex, is determined. If L1 and L2 are the same labels, then the measuring signal of both labels L1 and L2 present in the binding complexes is preferably determined at the point in time T2. Instead of measurement of the measuring signal of the bound labels, the measuring signal of the unbound label component, i.e., the label not present in the binding complex, may also be measured respectively or alternatively in the incubation batch.

Figure **2** illustrates the determination of high-dose hook samples, where the signal height at the point in time T2 is plotted as a function of the "T2 signal/T1 signal value."

The examples described below are used to illuminate individual aspects of this invention and are not to be understood as restrictive.

EXAMPLES:

Abbreviations used:

ADx	aminodextran
Biotin-X-NHS	sulfosuccinimidyl-6-(biotinamido)-hexanoate
BSA	bovine serum albumin
C-bead-ADx	aminodextran-coated chemiluminescer particles
C-bead-ADx-DxAl	aminodextran- and dextran aldehyde-coated chemiluminescer particles
CMO	carboxymethyloxime or carboxymethoxylamine hemihydrochloride
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DxAl	dextran aldehyde
EDAC	1-ethyl-3-(dimethylpropyl)carbodiimide
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3-N-(morpholino)propanesulfonic acid
$NaBH_3CN$	sodium cyanoborohydride
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NH_2	amino group
Sulfo-SMCC	sulfosuccinimidyl-4-(p-maleimidomethyl)cyclohexane 1-carboxylate
SC-ADx	single-coated aminodextran
S-bead-ADx	aminodextran-coated sensitizer particles
S-bead-DxAl	dextran aldehyde-coated sensitizer particles
S-bead-DxAl-SAV	streptavidine-coated S-bead-DxAl
STUT	O-(N-succinimidyl)-N,N,N'-tetramethyluronium tetrafluoroborate
TAPS	3-[N-tris-(hydroxymethyl)methylamino]propanesulfonic acid sodium salt
TAR	thioxene, anthracene, rubrene

Tris	tris(hydroxymethyl)aminomethane
$Zn(BF_4)_2$	zinc fluoroborate

Example 1: Coloring the latex particles

1) Chemiluminescer particles

Latex particles (Seradyn, catalog number 83000550100890, diameter approx. 0.2 µm) are suspended in a mixture of ethylene glycol, 2-ethoxyethanol and NaOH (65.4%, 32.2% and 2.3%) and stirred for 40 minutes at 95°C ± 3°C. Thioxene (203 mg/g particle), 1-chloro-9,10-bis(phenylethynyl)anthracene (16 mg/g particle) and rubrene (27 mg/g particle) are incubated at 95°C ± 3°C for 30 minutes in a 2-ethoxyethanol solution. The two batches are combined and incubated for 20 minutes while stirring. After these 20 minutes, the particle suspension is cooled down to 40°C ± 10°C. The colored particles are passed through a 43 micron mesh polyester filter and washed (Microgen Lab System). The particles are first washed with a solvent mixture of ethylene glycol and 2-ethoxyethanol (70% and 30%, 500 ml/g particle) and then with 10% ethanol (pH 10-11, 400 ml/g particle).

2) Sensitizer particles

Latex particles (Seradyn, catalog no. 83000550100890, diameter approx. 0.2 µm) are suspended in a solvent mixture of 2-ethoxyethanol, ethylene glycol and NaOH (65.4%, 32.2% and 2.3%) and stirred for 40 minutes at 95°C ± 3°C. The sensitizer dye (tert-butyl silicon phthalocyanine) is dissolved in benzyl alcohol (92 g dye/5 ml/g particle) and heated for 30 minutes at 95°C ± 3°C. After conclusion of the two incubation times, the two [sic] batches are combined and stirred for 20 minutes more. The particle suspension is cooled to 40°C ± 10°C and filtered through a 43 micron mesh polyester filter and then washed. First the particles are washed with an ethanol (700 ml/g particle) and then with 10% ethanol (pH 10-11, 600 ml/g particle).

Example 2: Production of aminodextran (ADx)

Several methods are known for preparing aminodextran. One method will be presented here. Hydroxypropyldextran (1 NH_2/7 glucose) is prepared by dissolving Dextran T-500

(Pharmacia, Uppsala, Sweden; 50 g) in 150 ml water in a glass flask with a stirrer and a dropping flask. 18.8 g $Zn(BF_4)_2$ is added to this solution and brought to a temperature of 87°C on a water bath. Epichlorohydrin (350 ml) is added by drops to the solution within 30 minutes while stirring. The mixture is stirred for 4 hours more at 85°C to 95°C, then cooled to room temperature. The resulting chlorodextran is precipitated by pouring the solution into 3 L methanol while stirring, then filtered and dried overnight in a vacuum oven.

The chlorodextran is dissolved in 200 ml water and added to 2 L 36% ammonia. The solution is stirred for 4 days at room temperature and then concentrated to 190 ml in a rotary evaporator. The concentrate is divided into two equal parts and poured slowly into 2 L methanol. The precipitate is filtered out and dried in a vacuum oven.

The dried precipitate is dissolved in 50 mM MOPS, pH 7.2 (12.5 mg/ml). The solution is stirred for 8 hours at room temperature, then cooled (4-8°C) and centrifuged for 45 minutes at 15,000 rpm in a Sorvall RC-5B centrifuge. To 10 ml of the supernatant is added 23.1 g sulfo-SMCC in 1 ml water. The mixture [is] incubated for 1 hour and used to coat the dyed chemiluminescer particles without further pretreatment (see example 4).

Example 3: Preparation of dextran aldehyde (DxAl)

Dextran aldehyde is prepared by stirring 400 g Dextran T-500 (Pharmacia, Uppsala, Sweden) into 1.5 L water in a 4-liter flask at 70°C. The solution is filtered and a $Zn(BF_4)_2$ solution (400 ml, 25 wt% in water, pH 1.8) is added under argon. Allyl-2,3-epoxypropyl ether is added by portions (3 × 500 ml, 8-10 ml/min) at a temperature of 70°C. For another 12-13 hours, the solution is stirred at 80°C under argon. Next the reaction mixture is cooled and poured into 6 liters of water. The dilute mixture is ultrafiltered using a Microgen tangential flow diafiltration system and concentrated to 1.0-1.5 L.

Allyloxydextran is then ozonylated while stirring. Ozone is generated by an ozonator and bubbled under pressure (9.0 psi) at a flow rate of 2 L/min through the allyloxydextran solution. 10 ml heptanol is added as a foam suppressant. After approx. 10 hours, the solution is cooled down to 10°C. 50 ml dimethyl sulfide is added under argon. After stirring continuously for 10 hours, dextran aldehyde is purified by Microgen ultrafiltration.

Example 4: Preparing aminodextran-coated TAR particles (C-bead-ADx)

1 ml (22 mg/ml) of the dyed chemiluminescer particles (example 1, paragraph 1) is mixed with 1 ml of an aminodextran solution (20 mg/ml, MW 500K, see example 2) in 0.05 [TrN: sic, M?] MES, pH 6.0 in the presence of 3.8 mg/ml EDAC. After an incubation time of 16 hours at room temperature (RT) in the dark, the particles are washed with 2 ml 0.05M MES, then with 6 ml 0.05M MES (1M NaCl, pH 6.0). The particles are placed in 1 ml 0.05M MES, pH 6.0 (22 mg/ml SC-ADx particles). The particles are washed by centrifugation (Sorval RC-5 B Plus or Eppendorf 5415C centrifuge) and resuspended by sonic treatment (Brenson Sonifier 450).

Example 5: Coating the C-bead-ADx with dextran aldehyde

1 ml of a dextran aldehyde solution (20 mg/ml, see example 3, MW 500K) and 1 ml of a 22 mg/ml C-bead-ADx solution (0.05M MES, pH 6) are incubated together with 2 mg/ml $NaBH_3CN$. After incubation for 20 hours at 37°C in the dark, the particles are washed twice with 5 ml MES buffer. The particles are then suspended in 0.5 ml 0.05M MES, 0.4% Tween 20, pH 6 (40 mg/ml C-bead-ADx-DxAl).

Example 6: Coating the C-bead-ADx-DxAl with anti-human IgG antibodies

Antibody-coated particles are prepared in an equal volume of an antibody solution (20 mg/ml anti-human IgG antibody in 0.05M MES, pH 5.0) and a particle suspension (40 mg/ml C-bead-ADx-DxAl in 0.05M MES, 0.4% Tween 20, pH 6) in the presence of 0.5 mg/ml $NaBH_3CN$. After incubation for 16 hours at room temperature, the remaining aldehyde groups are reacted with 0.08M CMO (carboxymethyloxime or carboxymethoxylamine) for 90 minutes at 37°C. The particles are then washed three to four times with a suitable buffer (Tris buffer, 1% BSA, 0.1% Zwittergent 3-14). The concentration of particles is 1 mg/ml after the last resuspension.

Example 7: Preparing dextran aldehyde-coated sensitizer particles (S-bead-DxAl)

The sensitizer particles from example 1, paragraph 2 are diluted in water to 2 mg/ml. Then the dilution is adjusted to a concentration of 18 mg/ml with 300 mM TAPS buffer, pH 9.0. Hydrazine (36.3 µL/g particle), STUT (0.456 g/g particle) and DMAP (23.2 mg/g particle)

are added. STUT (fresh in DMF, 10%) is added in four portions every 15 minutes. DMAP (fresh in DMF, 10%) is added after the first addition of STUT. After each addition of STUT, the pH is readjusted to 9.0. After one hour of stirring at room temperature, the particles are washed with a 10-fold volume of TAPS buffer, pH 9.0 (Microgen System).

The particles are placed in 1 mM TAPS buffer (20 mg/ml) and added slowly to 40 mg/ml dextran aldehyde in acetate buffer pH 5.0 with vigorous stirring. After 30 minutes, the reaction temperature is adjusted to 50°C and incubation is continued for 18 hours more while stirring. The particles are then washed with a 25-fold volume of water (Microgen System).

Example 8: Preparing streptavidine-coated sensitizer particles (S-bead-DxAl-SAv)

Dextran aldehyde-coated particles (S-bead-DxAl, 100 mg/ml) are added to a streptavidine solution (30 mg/ml 10 mM phosphate buffer pH 7). The solution is stirred for 1 hour at 37°C. Then sodium cyanoborohydride is added while stirring ($NaBH_3CN$ in water, 50 mg/ml, 2% of the total volume) and the total batch is stirred for another 40 to 72 hours at 37°C. Next 1M carboxymethoxylamine (CMO) in acetate buffer pH 5 is added (1.5% of the total batch) and incubated for 2 more hours. The particles are then washed with a 25-fold volume of a protein-free buffer (0.1M Tris, 0.3M NaCl, 25 mM EDTA, pH 8.2).

Example 9: Preparing rubella antigen-coated sensitizer particles

To prepare rubella antigen-coated sensitizer particles, dextrin aldehyde-coated particles (S-bead-DxAl) are reacted with L-lysine in the first step and with succinic anhydride in the second step. Then rubella antigen is coupled to the carboxyl groups. To 10 ml S-bead-DxAl particle (100 mg/ml in 50 mM MES, 0.2% Tween 20, pH 6.0) is added 10 ml of a lysine solution (40 mg/ml in MES, pH 6.0). 200 µL of a 10% Tween 20 solution is added. 2 ml of a freshly prepared sodium cyanoborohydride solution (25 mg/ml in H_2O) is added. The batch is incubated for 4 hours at 37°C. Then 2 ml of a freshly prepared sodium cyanoborohydride solution (25 mg/ml in H_2O) is again added. The reaction batch is incubated for 16 hours more at 37°C on a roll mixer. Next 10 ml borate buffer (200 mM, pH 9.0) is added, the mixture is centrifuged for 30 minutes (16,000 rpm, 10°C) and the supernatant is discarded. The pellet is placed in 15 ml borate buffer and resuspended. The batch is then sonified with a Sonifier 250 in a water bath (20 pulses, output 5 DC 50%).

10 ml (500 mg) of the S-bead-DxAl-lysine particles is mixed with 34.5 ml borate buffer. To the batch is added: 0.5 ml of a 10% Tween 20 solution and 1 ml of a succinic anhydride solution (10 mg in 100 ml DMSO). The batch is stirred for 2 hours at room temperature. 1 ml of a succinic anhydride solution (10 mg in 100 ml DMSO) is again added and incubated for 16 hours at room temperature with agitation. Next 10 ml MES buffer (50 mM MES, pH 5.0) is added, the mixture is centrifuged (30 minutes, 16,000 rpm, 10°C). The supernatant is discarded and the pellet is resuspended with 40 ml. The washing operation is repeated two or three times. In conclusion, the pellet is resuspended in approx. 8 ml MES buffer (50 mM, 0.1% Tween 20, pH 5.0) and homogenized with the Sonifier 250 (30 pulses, output 5, DC 50%).

Example 10: Preparation of biotinylated rubella antigen

To produce biotinylated rubella antigen, 6.5 ml of the rubella antigen solution (0.6 mg/ml rubella antigen in 0.1M $NaHCO_3$/0.25% Tween) is mixed with 650 µL biotin-X-NHS from the Pierce Company (1.2 mg/ml in DMSO) while stirring. After 22 hours, the batch is desalinated with 0.05M phosphate, 0.15M NaCl, 0.25% Tween 20, pH 7.6, using a desalination column from Pierce (Presto™).

Example 11: Performing an inventive immunoassay (high-dose hook assay)

10 µL sample is mixed with 50 µL of a particle suspension (C-bead-ADx-DexAl coated with anti-human IgG antibodies, 50 µg/ml, example 6) and 50 µL of a rubella antigen-coated particle suspension (S-bead-DxAl coated with rubella antigen 0.2 mg/ml, example 9) and 50 µL of a biotinylated rubella antigen solution (5 µg/ml) and incubated for 196 seconds (or 359 seconds) at 37°C. After this 196 seconds (359 seconds), the first signal is recorded (point in time T1). After another 81 seconds (and/or immediately after T1), 50 µL of the S-bead-DxAlSAv suspension (0.2 mg/ml) and 75 µL assay buffer (0.1M Tris buffer, 0.3M NaCl, 25 mM EDTA, 1 mg/ml BSA, pH 8.2) are added to the mixture and incubated for 264 seconds at 37°C. Then the second signal is recorded (point in time T2).

Example 12: Performing a standard assay

10 µL sample is mixed with 50 µL particle suspension (C-bead-ADx-DxAl coated with anti-human IgG antibodies, 50 µg/ml, example 6) and 50 µL biotinylated rubella antigen solution (5 µg/ml) and incubated for 277 seconds at 37°C. Then 100 µL of the S-bead-DxAl-SAv suspension (0.1 mg/ml) and 25 µL assay buffer are added to the mixture and incubated for 264 seconds at 37°C. Then the signal is recorded (point in time T2).

Table 1 compares the results of the various immunoassay formats

Sample dilution of the high-dose hook sample	"High-dose hook assay" (short T1) T1 signal (counts)	"High-dose hook assay" (long T1) T1 signal (counts)	"High-dose hook assay" T2 signal (counts)	Standard assay T2 signal (counts)
undiluted	21495	96651	1260815	1477950
1:2.5	10202	52762	1986820	2314755
1:5	6785	34393	759392	1283560
1:10	4901	18097	498505	718458
1:20	3964	11551	323291	456184
1:40	3273	7399	192937	251804
1:60	314	6257	127737	164181
1:100	2894	5461	71684	97251

The high-dose hook sample can now be determined on the basis of the signal height at the point in time T1 or on the basis of the signal height at the point in time T2, based on the signal ratio of T2 to T1. Figure 2 illustrates this relationship. With high-titer samples, the signal at the point in time T1 will here again increase until reaching the high-dose hook region. The value for [the ratio of] T2 to T1 will thus decline progressively, like the value for the signal height at the point in time T2. Short incubation times (T1) may be more advantageous to allow better identification of the high-dose hook sample (see also Figure 2).

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Claims

1. Method for quantitative or qualitative detection of an analyte A in a sample, with the sample being incubated with an analyte A-specific binding partner R1, which is associated with a solid phase, an analyte A-specific binding partner R2, which is associated with a label L1, and an analyte A-specific binding partner R3, which is associated with a label L2, and with the saturation of the analyte A binding sites of the binding partners R2 present in the incubation batch occurring at a higher analyte A concentration and/or at a later point in time during incubation in comparison with saturation of the analyte A binding sites of the binding partners R3 present in the incubation batch, characterized in that the L1-dependent measuring signal is either separated in time from the L2-dependent measuring signal or the L1-plus-L2-dependent measuring signal or is determined with the help of another measurement method.

2. Method according to Claim 1 for detection, prevention and/or reduction of the hook effect.

3. Method according to Claim 1 or 2, characterized in that (i) the sample is incubated with an analyte A-specific binding partner R1, which is associated with a solid phase, an analyte A-specific binding partner R2, which is associated with a label L1, and an analyte A-specific binding partner R3, which is associated with a member X of a specific binding pair; (ii) at a later point in time, label L2, which is associated with the binding pair member Y of a specific binding pair corresponding to X, is added to the assay batch; and (iii) the measuring signal is determined at a minimum of two points in time T1 and T2, with the earlier point in time T1 being at the latest shortly after addition of label L2, which is associated with binding pair

member Y, and the later point in time T2 being after addition of label L2, which is associated with binding pair member Y.

4. Method according to Claim 1 or 2, characterized in that (i) the sample is incubated with an analyte A-specific binding partner R1, which is associated with a solid phase, an analyte A-specific binding partner R2, which is associated with a label L1, and an analyte A-specific binding partner R3, which is associated with a member X of a specific binding pair; (ii) at a later point in time, label L2, which is associated with the binding pair member Y of a specific binding pair corresponding to X, is added to the assay batch; and (iii) the measuring signal of L1 and the measuring signal of L2 are determined with the help of different measurement methods.

5. Method according to any one of Claims 1 through 4, with the method being a heterogeneous or homogeneous sandwich assay.

6. Method according to any one of Claims 1 through 5, with R1 and R2, R1 and R3, R1, R2 and R3 or R2 and R3 being the same binding partners.

7. Method according to any one of Claims 1 through 6, with L1 and L2 being the same label.

8. Method according to any one of Claims 1 through 7, with the solid phase being a suspendable solid phase, preferably microparticles.

9. Method according to Claim 8, with the microparticles functioning as a label.

10. Method according to any one of Claims 1 through 9, with the binding partner R2 being associated with a suspendable solid phase, preferably microparticles.

11. Method according to Claim 10, with the microparticles representing the label L1.

12. Method according to any one of Claims 1 through 11, with components of a signal-forming system, which includes label L1 and/or label L2, being brought by the formation of the sandwich to a distance from one another that allows an interaction,

in particular an energy transfer between these components, and the extent of the interaction is measured.

13. Method according to Claim 12, with the signal-forming system comprising photosensitizers associated with microparticles and chemiluminescent substances associated with microparticles.

14. Use of an analyte A-specific binding partner R1, which is associated with a solid phase, an analyte A-specific binding partner R2, which is associated with a label L1, and an analyte A-specific binding partner R3, which is associated with a label L2, for detection, prevention and/or reduction of the hook effect in a method, in particular in a homogeneous sandwich assay for quantitative or qualitative detection of an analyte in a sample, and whereby the saturation of the analyte A binding sites of the binding partners present in the incubation batch takes place at a higher analyte A concentration and/or at a later point in time during incubation in comparison with the saturation of the analyte A binding sites of the binding partners R3 present in the incubation batch, characterized in that the L1-dependent measuring signal is either separated in time from the L2-dependent or L1-plus-L2-dependent measuring signal or is determined with the help of another measurement method.

15. Use of an analyte A-specific binding partner R1, which is associated with a solid phase, an analyte A-specific binding partner R2, which is associated with a label L1, an analyte A-specific binding partner R3, which is associated with a member X of a specific binding pair, and a label L2 which is associated with the binding pair member Y of a specific binding pair corresponding to X, and whereby the saturation of the analyte A binding sites of the binding partners R2 present in the incubation batch occurs at a higher analyte A concentration and/or at a later point in time during incubation in comparison with saturation of the analyte A binding sites of the binding partners R3 present in the incubation bath, for detection, prevention and/or reduction of the hook effect in a method, in particular in a homogenous sandwich assay, for quantitative or qualitative detection of an analyte A in the sample.

16. Assay kit for a heterogeneous or homogeneous sandwich assay for quantitative or qualitative detection of an analyte A in a sample, characterized in that it contains an analyte A-specific binding partner R1, which is associated with a solid phase, an analyte A-specific binding partner R2, which is associated with a label L1, and an analyte A-specific binding partner R3, which is associated with a label L2, preferably in separate containers, and whereby in the sandwich assay the saturation of the analyte A binding sites of the binding partners R2 present in the incubation batch takes place at a higher analyte A concentration and/or at a later point in time during incubation in comparison with saturation of the analyte A binding sites of the binding partners R3 present in the incubation batch.

17. Assay kit for a heterogeneous or homogeneous sandwich assay for quantitative or qualitative detection of an analyte A in a sample, characterized in that it contains an analyte A-specific binding partner R1, which is associated with a solid phase, an analyte A-specific binding partner R2, which is associated with a label L1, an analyte A-specific binding partner R3, which is associated with a member X of a specific binding pair and a label L2, which is associated with the binding pair member Y of a specific binding pair corresponding to X, preferably in separate containers, and whereby in the sandwich assay the saturation of the analyte A binding sites of the binding partners R2 present in the incubation batch takes place at a higher analyte A concentration and/or at a later point in time during incubation in comparison with the saturation of the analyte A binding sites of the binding partners R3 present in the incubation bath.

18. Assay kit according to Claim 17, with the analyte A-specific binding partner R2, which is associated with a label L1, and the analyte A-specific binding partner R3, which is associated with a member X of a specific binding pair, being present together in a container.

19. Assay kit according to any one of Claims 16 through 18 for performing the method according to any one of Claims 1 through 15.

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Abstract

The invention relates to methods for quantitative or qualitative detection of an analyte in a sample, with the sample being incubated with an analyte-specific binding partner R1, which is associated with a solid phase, an analyte-specific binding partner R2, which is associated with a label L1, and an analyte-specific binding partner R3, which is associated with a label L2, to prevent, reduce and/or detect the high-dose hook effect, and the L1-dependent measuring signal is either separated in time from the L2-dependent or L1-plus-L2-dependent measuring signal or is determined with the help of another measurement method.

Figure 1

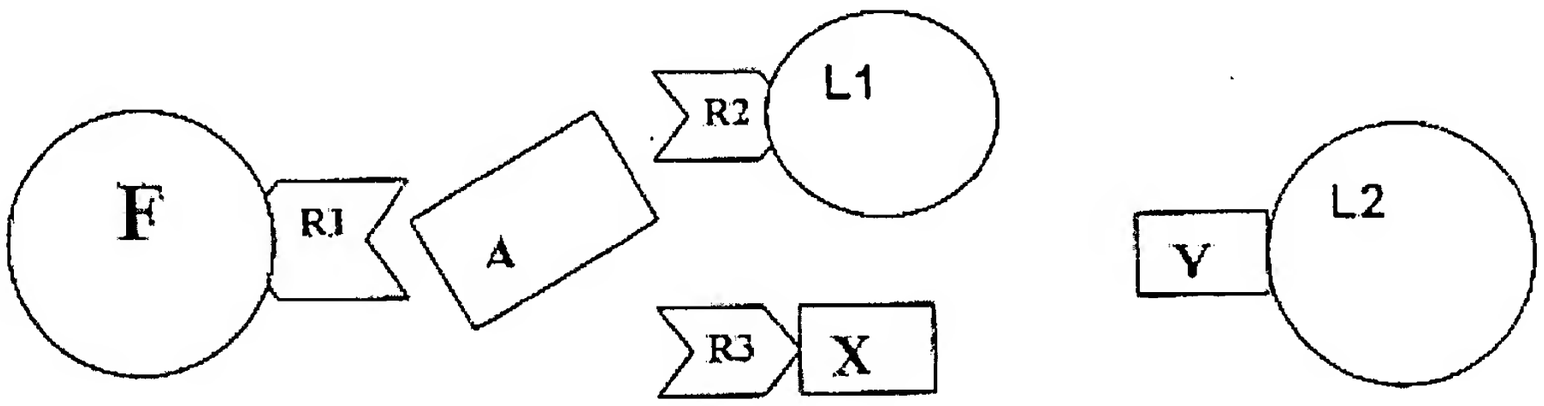

Figure 2